

Microinjection of Rap2B protein or RNA induces rearrangement of pigment granules in *Xenopus* oocytes

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Rap2B, a member of the ras superfamily of low-molecular-mass GTP-binding proteins, induced a characteristic rearrangement of the pigment granules in *Xenopus* oocytes following its microinjection, resulting in numerous unpigmented spots on the animal hemisphere. This phenomenon, termed 'mottling', was also induced by microinjection of *in vitro*-transcribed Rap2B RNA or of purified recombinant Rap2A. Following the microinjection of Rap2B, more than 90 % of the oocytes showed signs of mottling within 10 h. The time course of mottling paralleled the association of the recombinant Rap2B with an oocyte membrane

fraction. Like other members of the ras superfamily, Rap2B possesses a C-terminal CAAX motif that serves as a signal for post-translational processing. Mutation of the cysteine residue in the CAAX motif to serine prevents the association of Rap2B with oocyte membranes, and also prevents mottling. This result suggests that post-translational processing of Rap2B is required for the observed effect. Mottling was blocked by boiling Rap2B prior to its microinjection or by co-injection of the cytoskeletal reagent phalloidin.

INTRODUCTION

The Rap proteins are members of the ras superfamily of low-molecular-mass GTP-binding proteins. To date, four distinct proteins have been identified: Rap1A, 1B, 2A and 2B (Pizon et al., 1988a,b; Kitayama et al., 1989; Ohmstede et al., 1990; Lapetina et al., 1991). All share approximately 50 % amino acid sequence identity with the ras proteins, with the degree of similarity within the Rap group being greater than 60 %. Rap1A was initially identified by its ability to reverse the transformed phenotype of NIH 3T3 cells expressing the K-ras oncogene product (Kitayama et al., 1989). Rap1B is approximately 95 % identical at the amino acid level to Rap1A and, similarly, inhibits a ras-dependent pathway, i.e. meiotic maturation, in *Xenopus* oocytes (Campa et al., 1991). Work *in vitro* has led to speculation that the mode of action of Rap1A in transformation reversal may involve binding to the ras GTPase-activating protein (GAP), thereby blocking ras action (Frech et al., 1990).

Both Rap2A and Rap2B were identified by their similarity to ras. Using a *Drosophila* Dras3 probe, Pizon et al. (1988a) identified Rap2A from a human lymphoma cell line cDNA library. Rap2B was identified from a platelet cDNA expression library (Ohmstede et al., 1990) using a monoclonal antibody (M90) recognizing an epitope among amino acid residues 107–130 of H-ras (Lacal and Aaronson, 1986). Although as yet no activity *in vivo* has been ascribed to the Rap2 proteins, the localization of Rap2 to the specific granules of neutrophils has been reported recently (Maridonneau-Parini and de Gunzburg, 1992).

In this paper we show that the microinjection of recombinant Rap2A or Rap2B induces dramatic morphological changes in the pigmented animal hemisphere of *Xenopus* oocytes. These alterations, which we refer to as mottling, can also be triggered by microinjection of the RNA coding for Rap2B and can be blocked by co-injection with a microfilament stabilizing agent. The association of microinjected recombinant Rap2B with an oocyte membrane fraction suggests that the protein is modified by the oocyte. Preventing the processing of the recombinant

Rap2B by mutating the cysteine residue in the CAAX motif to serine blocks the association of Rap2B with oocyte membranes and also prevents mottling.

MATERIALS AND METHODS

Oocyte isolation and microinjection

Adult female *Xenopus laevis* were obtained from Xenopus I (Ann Arbor, MI, U.S.A.) and individual oocytes were isolated as described previously (Campa and Kilberg, 1989). Following an overnight incubation at 19 °C in modified Barth's medium (Colman, 1984), oocytes were microinjected with purified recombinant Rap2A or Rap2B protein or with *in vitro*-transcribed RNA. The microinjection volume was held constant at 50 nl. Variations in the quantity of Rap2 protein microinjected were achieved by adjusting the protein content of the solutions prior to microinjection. Protein concentrations were estimated using a dye-binding assay (Pierce) with BSA as standard.

Quantification of mottling

Following microinjection, oocytes were observed for signs of mottling using a Wild Leitz M5A binocular dissecting microscope at 15× magnification. Oocytes were scored positive for mottling with the appearance of well-defined unpigmented spots located on the animal hemisphere, similar to those shown in Figure 1(a), 4 h after microinjection. The appearance of the spots is characteristic of the Rap2 effect and was not observed after microinjection of other members of the ras superfamily of proteins (see the Results section for details). A total of 10–20 oocytes were used for each condition.

Protein purification

The construction of a plasmid for expression of Rap2B in *Escherichia coli* and purification of the recombinant protein were as described previously (Ohmstede et al., 1990; Molina y Vedia

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et al., 1990). The purity of the protein used for microinjection was estimated to be greater than 90% by Coomassie Blue staining.

The Rap2A coding sequence was subcloned into a *Bam*HI/*Eco*RI-digested pGEX-2T vector (Pharmacia) and the product was expressed as a fusion protein. Expression and purification of the glutathione S-transferase–Rap2A protein were as described in Ausubel et al. (1989). Thrombin (1%, w/w) was incubated with the purified fusion protein for 1 h at 25 °C to liberate recombinant Rap2A.

A mutant Rap2B, in which the cysteine at position 180 is replaced by a serine, was generated using a standard PCR protocol following the manufacturer's directions with the oligonucleotide primers 5'-CCCGGGGGATCCATGAGAGAGTACAAAGTG-3' and 5'-CCCGGGGAATTCTTATCAGAGGATCACGGAGGC-3'. The PCR-generated fragment was gel-purified, digested with *Bam*HI/*Eco*RI and subcloned into *Bam*HI/*Eco*RI-digested pGEX-2T as described above. The purified protein was shown not to incorporate a prenyl moiety *in vitro* (results not shown).

In vitro transcription of Rap2B cDNA

The full-length cDNA of Rap2B isolated from a human platelet cDNA library was subcloned into the *Eco*RI site of pGEM-3Z. The orientation of the insert was established by sequencing and restriction digest analysis. Run-off transcripts were prepared by digesting the plasmid at the unique *Nar*I site located 3' to the insert. The linearized plasmid was transcribed with SP6 polymerase according to the manufacturer's instructions (Promega). To confirm that the transcription product encoded Rap2B of the expected molecular mass, the RNA was translated in a methionine-deficient rabbit reticulocyte lysate system (Promega). A standard 50 µl reaction containing 2 µg of RNA and 4 µl of [³⁵S]methionine (1200 Ci/mmol; 10 mCi/ml) was incubated for 1 h at 30 °C. A 10 µl aliquot of the reaction mixture was subjected to SDS/PAGE and the dried gel used to expose Kodak XAR film overnight. An anti-sense transcript was generated by digesting the vector at the *Hind*III site located 5' to the insert and transcription was initiated by T7 polymerase.

Oocyte membrane isolation

At various times after microinjection, an oocyte membrane fraction was prepared based on the procedure of Colman (1984). Thirty oocytes were homogenized in 1 ml of ice-cold T-150 buffer (150 mM NaCl, 20 mM Tris/HCl, pH 7.6, 10 mM MgCl₂, 10 µg of aprotinin/ml, 10 µg of leupeptin/ml, 1 mM phenylmethanesulphonyl fluoride) containing 10% (w/v) sucrose. The homogenate was layered over a step gradient consisting of 1 ml of 20% and 1 ml of 50% (w/v) sucrose in T-50 buffer (same as T-150 buffer, except that it contains 50 mM NaCl). The tubes were centrifuged at 4 °C for 35 min at 15000 *g* in a TST 60.4 rotor (Sorvall). The 20%/50% sucrose interface was then transferred to a clean centrifuge tube, diluted several-fold with T-150 buffer and centrifuged at 4 °C for 60 min at 200000 *g* in a fixed-angle rotor. The resulting membrane pellet was solubilized in 200 µl of 100 mM NaCl, 20 mM Tris/HCl, pH 7.6, 1% (v/v) Triton X-100 and proteinase inhibitors and the extract was cleared by centrifugation at 12000 *g* in a microcentrifuge for 15 min at 4 °C.

Preparation of oocyte extracts

Total oocyte extracts were prepared by homogenizing oocytes in buffer containing 100 mM NaCl, 20 mM Tris/HCl, pH 7.6, 1% (v/v) Triton X-100 and proteinase inhibitors. Oocytes were

homogenized using 10 µl of homogenization buffer per oocyte. The homogenates were cleared by centrifugation at 12000 *g* for 5 min at 4 °C in a microcentrifuge and the supernatant fractions were stored at –80 °C until use.

Immunoprecipitation

For immunoprecipitation of microinjected Rap2B, solubilized membrane proteins from 30 oocytes were diluted into 450 µl of RIPA buffer [150 mM NaCl, 50 mM Tris/HCl, pH 8.0, 1% (v/v) Nonidet P-40, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, and proteinase inhibitors] and 6 µl of anti-Rap2 serum (Winegar et al., 1991) was added. After incubation for 60 min on ice, Protein A–Sepharose (CL4B; Pierce) was added and the incubation continued for an additional 45 min with rotation. The pellet was washed three times with RIPA buffer and once with 10 mM Tris/HCl, pH 7.5, and then boiled for 4 min in SDS/PAGE sample buffer. Electrophoresis was carried out as described below.

Electrophoresis and Western blotting

Proteins were separated via SDS/PAGE on a 12% (w/v) polyacrylamide gel according to the method of Laemmli (1970). The proteins in the gel were then electrophoretically transferred to nitrocellulose and the excess sites blocked by incubation for 30 min in buffer A [200 mM NaCl, 50 mM Tris/HCl, pH 7.6, 0.02% (w/v) NaN₃, 0.1% (w/v) BSA, 0.1% (w/v) poly(ethylene glycol) (approx. molecular mass 15–20 kDa)] containing an additional 3% (w/v) BSA. The nitrocellulose was then incubated for 60 min at room temperature in primary antibody (anti-Rap2 serum) diluted in buffer A. The blot was washed three times for 5 min each in buffer A, and then diluted alkaline phosphatase-conjugated secondary antibody was added. Following a 45 min incubation, the blot was washed as before and immunoreactive proteins visualized with Nitro Blue Tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate.

RESULTS

Mottling in oocytes induced by microinjected recombinant Rap2 protein

Recombinant Rap2B was purified from *E. coli* (Molina y Vedia et al., 1990) and microinjected into *Xenopus* oocytes. Approx. 4 h after microinjection, a distinctive rearrangement of the pigment granules of the animal hemisphere (hereafter referred to as mottling) was observed. The Rap2B-injected oocytes initially developed a series of small (30–40 µm diameter) unpigmented spots on the surface of the animal hemisphere. These spots gradually increased in size and number over the next few hours until the entire hemisphere was mottled (Figure 1a). The time course of mottling determined using oocytes from several different female frogs was remarkably consistent (Figure 2a). More than 90% of the oocytes showed signs of mottling within 10 h after microinjection. The relative potency of Rap2B for inducing mottling is illustrated in Figure 2(b). As little as 5 ng of the protein caused mottling in a small percentage of oocytes. Based on an estimated oocyte water volume of 0.5 µl (M. J. Campa, unpublished work), this represents an intracellular Rap2B concentration of approx. 500 nM. Larger amounts of Rap2B induced the effect in a higher proportion of oocytes and with increasing rapidity. Boiling the Rap2B prior to its microinjection eliminated the effect.

The microinjection into oocytes of other members of the ras protein superfamily, such as Rap1B or [Leu⁶¹,Ser¹⁸⁶]p21^{H-ras}, did

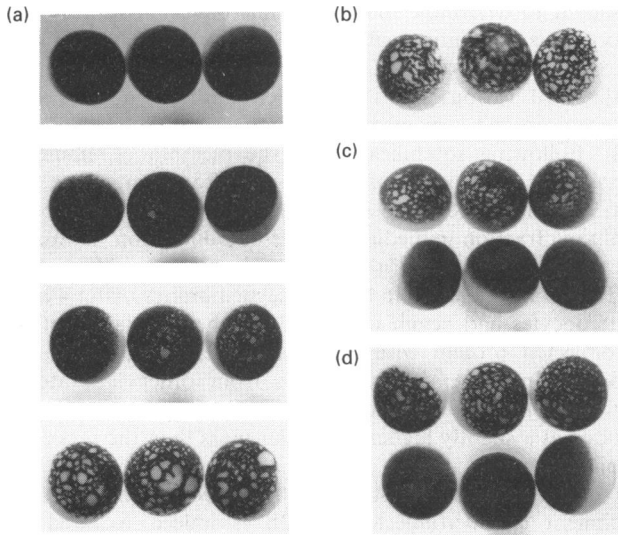


Figure 1 Rap2-induced mottling in oocytes

Oocytes were isolated and microinjected as described in the Materials and methods section. (a) Following the microinjection of 20 ng of recombinant Rap2B, oocytes were observed for signs of mottling and photographed. In the top photograph are uninjected oocytes. Pictured below (from top to bottom) are oocytes 4, 5 and 8 h after microinjection. (b) Oocytes were microinjected with 20 ng of recombinant Rap2A and photographed 8 h later. (c) Oocytes were microinjected with 25 ng of *in-vitro*-synthesized Rap2B RNA transcribed in the sense (top row) or anti-sense (bottom row) direction and photographed 24 h later. (d) Oocytes were microinjected with 20 ng of recombinant Rap2B alone (top row) or in combination with 500 μ M phalloidin (bottom row) and photographed 8 h later. Photography was carried out using a Wild Leitz stereo microscope (15 \times) equipped with a Nikon camera.

not induce mottling. Likewise, the microinjection of Harvey or Kirsten ras proteins with valine at position 12 triggered germinal vesicle breakdown but no evidence of mottling (results not shown). The ability of Rap1B to block [Val¹²]p21^{H-ras}-induced oocyte meiotic maturation has been reported previously (Campa et al., 1991). In similar experiments, Rap2B was shown to have no such activity, nor was an acceleration of ras-induced germinal vesicle breakdown observed. These data are in agreement with those of Jimenez et al. (1991) reporting a lack of interference with ras transformation for Rap2 expressed in murine fibroblasts. Furthermore, Rap2 had neither growth-promoting nor growth-inhibiting activity in the same system.

Given the high degree of sequence similarity (approx. 90%) between the Rap2 proteins, we sought to determine if microinjected Rap2A would elicit the same morphological changes as Rap2B. As illustrated in Figure 1(b), Rap2A induced a pattern of mottling essentially identical to that caused by the 2B protein.

Mottling in oocytes induced by Rap2B RNA

Although the Rap2B protein used for microinjection was estimated to be at least 90% pure by Coomassie Blue staining, it is conceivable that mottling is the result of a contaminating protein in the preparation. In order to rule out this possibility, we microinjected Rap2B RNA that had been transcribed *in vitro*. As can be seen in Figure 1(c), Rap2B RNA duplicated the effect of the purified protein, while an equivalent amount of Rap2B anti-sense RNA resulted in no observable change in the appearance of the oocytes. No obvious difference in the mottling pattern was observed between the RNA- and the protein-injected oocytes. The presence of the Rap2B protein in the RNA-injected oocytes

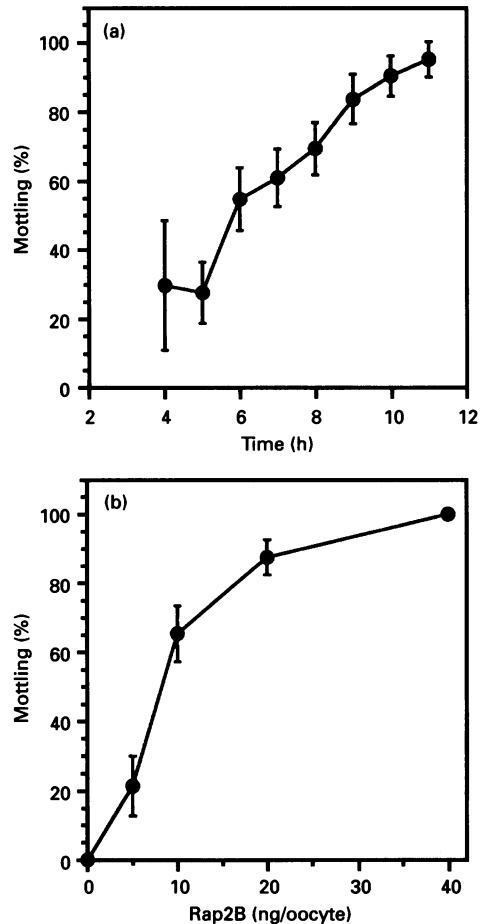


Figure 2 Mottling in oocytes induced by microinjected Rap2B protein

Purified recombinant Rap2B was microinjected into *Xenopus* oocytes and mottling was monitored as described in the Materials and methods section. (a) Oocytes were microinjected with 20 ng of Rap2B and observed for signs of mottling. The data point at 4 h is the mean \pm S.E.M. from three independent experiments; all other data points are the means \pm S.E.M. of 8–10 experiments. (b) Oocytes were microinjected with the indicated quantities of Rap2B and observed for mottling. The percentage mottling was determined for oocytes receiving 0, 5, 10 or 20 ng Rap2B at the time point when 100% of those receiving 40 ng of the protein were positive for mottling. Each point represents the mean \pm S.E.M. of five experiments.

was verified by immunoprecipitation and immunoblotting using anti-Rap2 serum (Figure 3).

Association of microinjected Rap2B with an oocyte membrane fraction

Members of the ras superfamily of low-molecular-mass GTP-binding proteins possess a CAAX motif at the C-terminus (Hancock et al., 1989). This amino acid sequence motif serves as a signal for the post-translational modification of the proteins that, in general, involves isoprenylation, C-terminal proteolysis and O-methylation. Translocation of the protein from a soluble to a particulate fraction occurs concomitantly with post-translational modification, consistent with the notion that the isoprenoid moiety functions, in part, as a membrane anchor (Barbacid, 1987; Jackson et al., 1990). Processing of microinjected recombinant ras protein by *Xenopus* oocytes has been shown to occur (Birchmeier et al., 1985). Given that the amount of time required for the processing of exogenous ras in oocytes was similar to that required for the commencement of mottling,

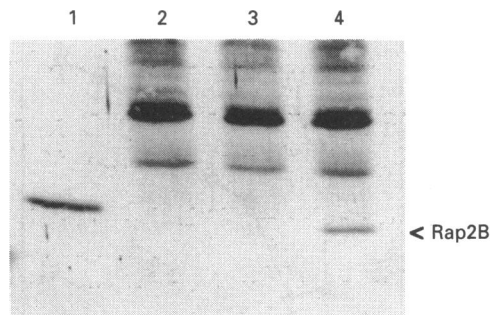


Figure 3 Translation of *in-vitro*-transcribed Rap2B RNA in oocytes

Oocytes were microinjected with 25 ng of Rap2B RNA transcribed in the anti-sense (lane 3) or sense (lane 4) orientation. After incubation for 24 h, oocytes were observed for mottling, photographed (Figure 1c) and homogenized. Immunoprecipitation was performed on the extracts using anti-Rap2 serum. Immunoprecipitated proteins were analysed by SDS/PAGE and immunoblotting using anti-Rap2 serum. Lane 1 contains approx. 2.5 ng of recombinant Rap2B purified from *E. coli*, and lane 2 contains the immunoprecipitated proteins from uninjected oocytes. All immunoprecipitations were carried out using the extracts from 15 oocytes. At the time of homogenization, all oocytes that had been microinjected with sense RNA were mottled, while those left uninjected or receiving anti-sense RNA appeared normal.

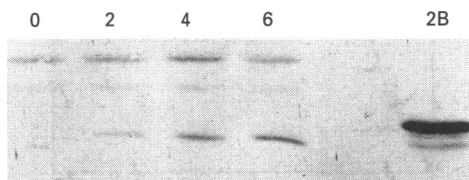


Figure 4 Association of microinjected Rap2B with an oocyte membrane fraction

Oocytes were microinjected with 40 ng of recombinant Rap2B. Immediately following microinjection (lane 0) or after 2, 4 or 6 h as indicated, the oocytes were homogenized and a membrane fraction was obtained as described in the Materials and methods section. The solubilized membrane proteins were analysed by immunoprecipitation and immunoblotting using anti-Rap2 serum. The numbers of oocytes out of 30 displaying signs of mottling after 0, 2, 4 and 6 h were 0, 7, 30 and 29 respectively. The lane labelled '2B' contains 75 ng of recombinant Rap2B purified from *E. coli*.

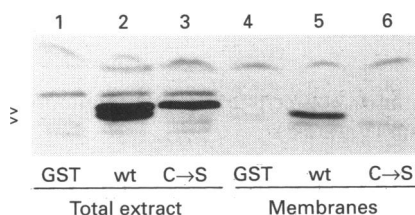


Figure 5 Membrane association of microinjected Rap2B containing a Cys→Ser mutation

Using PCR, the cysteine residue at position 180 of Rap2B was changed to serine and the glutathione transferase-[Ser¹⁸⁰]Rap2B fusion protein was purified using glutathione affinity chromatography. Following removal of the GST moiety via thrombin cleavage, the mutated protein was used for microinjection. Oocytes were microinjected with 20 ng of the wild-type protein (lanes 2 and 5) or of the Cys→Ser mutant (lanes 3 and 6). As a control, 40 ng of GST was microinjected alone (lanes 1 and 4). When all of the oocytes receiving the wild-type Rap2B had begun to mottle, total oocyte extracts or membrane fractions were prepared as described in the Materials and methods section. Oocyte proteins were analysed by SDS/PAGE and immunoblotting using anti-Rap2B serum. Lanes 1–3 each contain the protein from 1.5 oocytes, and lanes 4–6 each contain the membrane-associated proteins from five oocytes. Abbreviations: GST, glutathione transferase; wt, wild-type; C→S, Cys→Ser mutated protein. The unprocessed (upper arrowhead) and processed (lower arrowhead) Rap2B proteins are indicated to the left of the Figure.

we sought to determine the fate of microinjected Rap2B with respect to its association with oocyte membranes. Oocytes were microinjected with 40 ng of Rap2B and monitored for signs of mottling. At various times after microinjection, oocytes were homogenized and a membrane fraction was isolated (Colman, 1984). Following solubilization of the membranes, immunoprecipitation was performed using anti-Rap2 antiserum. As illustrated in Figure 4, no Rap2B was detected in the oocyte membrane fraction immediately after microinjection. However, after 2 h, approx. 20% of the oocytes showed signs of mottling and Rap2B was detected in the oocyte membranes. After 4 h, all of the oocytes had begun to mottle and the association of the microinjected protein with the membrane fraction had also increased. In agreement with data from other laboratories (Gutierrez et al., 1989), the unprocessed recombinant protein migrated at a slightly higher molecular mass than the processed protein (Figure 4).

In order to better substantiate the notion that membrane attachment of microinjected Rap2B is indeed required for mottling, we generated a mutant Rap2B in which the cysteine residue of the CAAX box was changed to serine, as described in the Materials and methods section. Other laboratories have shown that this mutation prevents processing and subsequent membrane localization of the protein (Hancock et al., 1989). Microinjection of the mutated protein into oocytes did not cause mottling, whereas the wild-type protein did. After the oocytes that had been microinjected with wild-type Rap2B had become mottled, total homogenate and membrane fractions from all oocytes were analysed by SDS/PAGE and immunoblotting for the presence of Rap2. As illustrated in Figure 5, no Rap2B was detected in the control (GST-injected) oocytes in either the total extract or the membrane fraction (lanes 1 and 4). However, both the wild-type Rap2B and the Cys→Ser mutant were present in the extract (lanes 2 and 3). In contrast, only the wild-type protein was associated with the oocyte membranes (lane 5). Moreover, the wild-type Rap2B migrated as a doublet in the total homogenate (lane 2), with the lower band, representing the processed species, migrating at the same molecular mass as the protein in the membrane fraction (lane 5).

Inhibition of mottling by phalloidin

The microinjection of phalloidin, a toxic peptide from *Amanita phalloides* (Dancker et al., 1975), into cultured cells results in marked alterations in the structure and organization of actin microfilaments (Wehland et al., 1977). When microinjected at a concentration of 200 μ M, phalloidin caused the aggregation of actin polymers that were easily visible using immunofluorescence microscopy (Wehland et al., 1977). At concentrations between 200 and 500 μ M, the toxin decreased cell locomotion, and at 1 mM it resulted in a cessation of cell growth (Wehland et al., 1977). In oocytes, phalloidin inhibited Rap2B-induced mottling in a dose-dependent manner (Figure 6). Total inhibition was observed with 500 μ M phalloidin. Oocytes receiving the combination of Rap2B and 500 μ M phalloidin showed no signs of mottling, while those receiving Rap2B alone were heavily mottled. The appearance of the oocytes 8 h after Rap2B microinjection with or without phalloidin is shown in Figure 1(d). Phalloidin alone did not induce any noticeable alteration in oocyte pigmentation (results not shown). The levels of phalloidin that were effective in preventing Rap2B-induced mottling represent intra-oocyte concentrations of approx. 25–50 μ M. If a similar dilution of the microinjected sample is assumed in the case of cultured cell microinjection, the effective doses of phalloidin in the two systems are comparable.

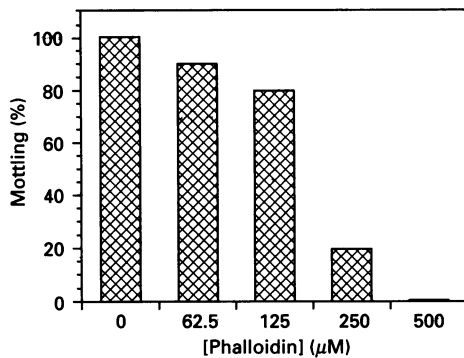


Figure 6 Inhibition of Rap2B-induced mottling by phalloidin

Oocytes were microinjected with a combination of 20 ng of recombinant Rap2B and the indicated concentrations of phalloidin. Scoring for mottling was as described in the Materials and methods section. The values shown were obtained 8 h after microinjection.

DISCUSSION

The results presented above demonstrate a characteristic rearrangement of the pigment granules in oocytes, termed mottling, following the microinjection of recombinant Rap2 proteins. Although both Rap2A and Rap2B caused mottling, the bulk of the experiments were performed using the Rap2B protein. As indicated by the altered mobility of microinjected Rap2B in SDS gels and its association with an oocyte membrane fraction, it is clear that the recombinant protein is processed by oocytes (Figures 4 and 5). Moreover, C-terminal processing appears to be required for Rap2B to cause mottling, as a processing-defective mutant is unable to do so (Figure 5). A plausible explanation for mottling is that the microinjected Rap2B competes with endogenous CAAX-motif-containing proteins for processing, thereby inactivating proteins required for the maintenance of oocyte pigmentation. However, this possibility was ruled out by our finding that the C-terminal 20 amino acid residues of Rap2B or Rap2A were incapable of inducing mottling when microinjected into oocytes (results not shown) although being of sufficient length to serve as substrates for protein prenyltransferases (Reiss et al., 1990).

A separate class of low-molecular-mass GTP-binding proteins with which morphological alterations have been associated is the rho family. The rho proteins are substrates for the C_3 ADP-ribosyltransferase from *Clostridium botulinum* (Sekine et al., 1989). Inactivation of endogenous rho in cultured cells by microinjected C_3 causes disruption of actin filaments and gross morphological changes that are observable at the light microscope level (Paterson et al., 1990). In experiments analogous to those in oocytes described above, Mohr et al. (1990) have reported morphological alterations of the *Xenopus* oocyte animal hemisphere following the microinjection of [Val¹⁴]p21^{rhoA}. The morphological perturbations elicited by the rho protein are, however, visibly quite distinct from those caused by Rap2B (compare Figure 1c of Mohr et al., 1990, with Figure 1a of the present paper). Moreover, the effect of [Val¹⁴]p21^{rhoA} proved to be dependent upon the presence of progesterone in the incubation medium, while Rap2B displayed no such dependency (results not shown). On the other hand, exposure of oocytes to 10 μM progesterone immediately following Rap2B microinjection resulted in meiotic maturation of the oocytes with no evidence of mottling. The rho effect appeared also to require that the protein possess an activating mutation, while Rap2B was active in the

native state. The effect of an analogous mutation on Rap2B activity in oocytes was not examined.

An interesting finding is that phalloidin, a toxin from *Amanita phalloides*, blocks Rap2B-induced mottling in oocytes (Figures 1d and 6). Phalloidin has been shown to stabilize existing microfilaments and to promote the polymerization of monomeric actin (Wieland, 1977; Wehland et al., 1977; Dancker and Hess, 1990). Although the inhibition of mottling by phalloidin by no means proves that mottling is a result of a direct interaction of Rap2B with oocyte microfilaments, it does suggest that some component of the oocyte cytoskeleton is involved in the phenomenon. A plausible interpretation of the inhibitory effect of phalloidin is that mottling is a result of microfilament depolymerization. This, however, implies that any reagent leading to an increase in the proportion of monomeric actin should produce mottling. The cytochalasins, a family of toxic metabolites produced by certain species of moulds, cause microfilament depolymerization by preventing the addition of actin monomers to the ends of existing microfilaments (Lin et al., 1980). In oocytes, although both cytochalasins B and D caused perturbations in the pigmented animal hemisphere in the absence of Rap2B, the effect was quite distinct from Rap2B-induced mottling (results not shown). Microinjection of Rap2B immediately preceding exposure to cytochalasin did not result in mottling *per se*, but magnified the effect of the toxin. In the light of this result, we believe mottling to be the result of a cytoskeletal restructuring that, by definition, involves a depolymerization/repolymerization sequence. Phalloidin prevents the initial depolymerization step while cytochalasin blocks the subsequent repolymerization. Once again, we stress that this notion does not necessarily imply a direct interaction of Rap2B with oocyte microfilaments, but simply that microfilament reorganization is likely to be involved in mottling.

Regardless of the mode of action by which the Rap2 proteins cause mottling in *Xenopus* oocytes, the results presented above represent the first report of a biological effect associated with this sub-class of low-molecular-mass GTP-binding proteins. The localization of Rap2 to the cytoplasmic face of the specific granules of human neutrophils was demonstrated recently (Maridonneau-Parini and de Gunzburg, 1992) and suggests a role for this family of proteins in the trafficking of vesicles in transit from cytoplasmic locations to the plasma membrane. Mottling may reflect an analogous function of Rap2 in oocytes, a cytoskeletal reorganization being required for vesicle movement. Attempts to detect endogenous Rap2 in oocytes have been unsuccessful, however, possibly indicating that Rap2 function in *Xenopus* may not be required until a later stage of development, with the effect on oocyte pigmentation resulting from the premature presence of Rap2.

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